Urol Res (1998) 26:11–15

ORIGINAL PAPER

L. Z. Solomon · A. M. Jennings · M. C. Hayes
P. S. Bass · B. R. Birch · A. J. Cooper

Is gamma-linolenic acid an effective intravesical agent for superficial bladder cancer? In vitro cytotoxicity and in vivo tolerance studies

Received: 29 April 1997 / Accepted: 1 August 1997

Abstract The essential fatty acid gamma-linolenic acid (GLA) is an effective cytotoxic agent when applied topically and for prolonged periods to tumour cells. Topical application, by intravesical therapy, is firmly established in the treatment of superficial bladder cancer. However, this form of therapy is limited to a maximum duration of 2 h. At such a short drug exposure time, does GLA retain its cytotoxicity? We have examined this question by exposing the superficial bladder cancer cell lines MGH-U1 and RT112 to meglumine-GLA (MeGLA) for time intervals ranging from 30 min to 2 h, at drug concentrations ranging from 1000 to 1.95 μg/ml. The MTT viable biomass assay was used to assess cell kill. Greater than 90% inhibition was observed at a concentration of 125 μg/ml (IC > 90), at 2 h drug exposure. At shorter drug exposure times, higher drug concentrations were needed to induce the same effect. At 1 h drug exposure, the IC > 90 was recorded at 500 μg/ml. In vivo intravesical tolerance studies were conducted in rats. Rats exposed to 2.5 mg/ml MeGLA intravesically for 2 h or less remained well and bladder histology showed minimal changes. This study confirms that GLA retains its cytotoxicity at short drug exposure times and is well tolerated by normal bladder mucosa in vivo. Bladder mucosa tolerated >10 × the concentration required for the IC > 90 in vitro. MeGLA is therefore a feasible intravesical agent for superficial bladder cancer.

Key words Gamma-linolenic acid · Superficial bladder cancer · Intravesical therapy · Cytotoxicity

L. Z. Solomon(✉) · A. M. Jennings · M. C. Hayes
P. S. Bass · B. R. Birch · A. J. Cooper
Department of Urology, Southampton University Hospitals NHS Trust, Southampton, S016 6YD, UK

Introduction

Although most of the in vitro work on the anti-tumour effect of fatty acids has been done in the last 15 years it was previously known that polyunsaturated fatty acids can decrease proliferation of both benign and malignant cells such as glioma [12]. The initial proposal that deficiency of gamma-linolenic acid (GLA) in malignant cells may be pivotal to the metabolic abnormalities of cancer cells [9], prompted experiments where fatty acids such as GLA were added to malignant cells in culture.

Such studies since 1982 have shown that polyunsaturated fatty acids such as GLA have a direct cytotoxic effect on malignant cells at high concentrations or a growth inhibitory effect at low concentrations. This effect is not organ- or tissue-specific as a wide variety of human malignant cells of both epithelial and non-epithelial origin have been shown to be susceptible to the growth inhibitory effect of fatty acids in vitro. These include osteogenic sarcoma [3], oesophageal carcinoma [10] and prostate carcinomas [2]. The selectivity of the action of GLA on neoplastic cells is generally agreed [7], although some authors believe the effect to rely on differential cell turnover rates rather than transformation or malignancy, for example [16]. Some studies also postulate an enhancement of uptake of conventional cytotoxic agents after pretreatment with GLA, for example [6]. Indeed multidrug resistant (MDR) cell lines are more sensitive to the polyunsaturated fatty acids [14].

The in vivo efficacy of GLA is more controversial [4, 10]. There are identifiable problems with parenteral delivery of the drug [4] and it is apparent that the best anti-tumour effect is obtained when the route of administration is essentially topical [5]. Intravesical chemotherapy for superficial bladder cancer involves direct contact between the tumour and the cytotoxic agent.

No in vitro studies have assessed the cytotoxic effects of high concentrations of GLA after short exposure, neither has the intravesical tolerance of the drug been

established. GLA is theoretically an attractive agent for intravesical therapy as it is cytotoxic when delivered topically, has a selective affinity for malignantly transformed cells and it by-passes the problem of multidrug resistance [17].

This study investigates the cytotoxic effects of exposing two urothelial cancer cell lines (and an MDR derivative) to high concentrations of meglumine-GLA (MeGLA) for 2 h or less. In vivo tolerance of rat bladders to MeGLA is also described. Conventionally the GLA formulation of choice for topical application is MeGLA, whereas the lithium salt is used parenterally.

Materials and methods

The transitional carcinoma cell line MGH-U1 and a doxorubicin-resistant clone were a gift from University College, London (UCL). The mitomycin-C-resistant clone was derived in our laboratories from the parental line by chronic exposure to drug. Both resistant clones were of the classical MDR phenotype and were cross-resistant to epirubicin. RT112, a transitional carcinoma cell line, and its cisplatin-resistant clone were also a gift from UCL. These human cell lines have been characterised by Masters et al. [11]. The cisplatin-resistant cells were not MDR and showed normal epirubicin uptake. These anchorage-dependent cell lines were cultured using Dulbecco's modification of Eagle's minimal essential medium, supplemented with 10% fetal calf serum and 1% Sigma A9909 antimicrobial cocktail (DMEM) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured using trypsin-EDTA.

MeGLA was obtained courtesy of Dr. Richard Bryce of Scotia Pharmaceuticals, Stirling, UK. The drug was supplied in 1-ml vials at a concentration of 5 mg/ml and stored at +4°C. In this preparation, GLA is esterified with methyl glutamine.

For experimental use, cell suspensions were counted (haemocytometer) and adjusted to achieve a concentration of 100 000 cells/ml. One hundred and twenty-five microlitres was seeded into each well of culture-grade 96-well microtitre plates and incubated as above for a period of 24 h.

The medium was then thrown off and replaced with 100 µl DMEM now containing MeGLA at doubling dilutions from 1000 to 1.95 µg/l. Incubation with the drug varied between 30 min and 2 h. Following this period, the medium was again tipped off and 150 µl per well of fresh drug-free medium added. The plates were either incubated for 5 days or assayed immediately.

The residual viable biomass at the end of the experiment was estimated by an MTT assay similar to that described by Freshney [8]. Twenty-five microlitres of MTT (5 mg/ml) was added per well and the plates re-incubated for a period of 4 h. The supernatant was thrown off and 150 µl dimethyl sulphoxide added per well (without washing) prior to colorimetry at 570 nm using a microtitre plate reader.

The in vivo drug tolerance was assessed using a female rat model. Twelve adult female Fischer rats weighing 170–200 g were included in the study. Surgical anaesthesia with good muscle relaxation was achieved for at least 60 min by making up a mixture of 1 part midazolam + 1 part Hypnorm (Janssen) + 2 parts sterile water and administering 2.7 ml/kg via the intraperitoneal route. The rats were subsequently catheterised *per urethram* using an 18-gauge drawing up needle (Becton Dickinson). Each rat had 0.5 ml of MeGLA instilled at a concentration of 2.5 mg/ml (diluted in phosphate-buffered saline). Catheterisation was maintained for 60 min post-instillation and the rats were held in a head-down position to prevent leakage. Two rats had phosphate-buffered saline instilled as controls. Urine microscopy was performed daily on samples obtained from the rats by suprapubic stimulation. The bladders were harvested at days 1, 2, 5, 7 and 14. During harvesting 0.5 ml of a solution containing 10% formal saline was instilled into the bladder and a ligature applied around the urethra. The bladders were then processed and stained using H&E prior to histological assessment by a single consultant pathologist.

Results

Experiments on duration of drug exposure

Figure 1 illustrates experiments in which the effect of varying the drug exposure times has on the cytotoxicity profile of MeGLA. MGH-U1 cells incubated with MeGLA for a period of 30 min demonstrated an IC > 90 at 1000 µg/l only. The IC > 90 at 1 h of drug exposure was recorded at 500 µg/ml. At 90 min of drug exposure, the IC > 90 was attained at a drug concentration of 250 µg/ml. The IC > 90 at 2 h of drug exposure was recorded at 125 µg/ml. At 2 h, 62.5 µg/ml of Me-GLA is cytotoxic to 50% of the MGH-U1 cells. At dosages of 62.5 µg/ml and 31.25 µg/ml MeGLA, the optical density exceeds that of controls but this is neither a consistent finding nor is it statistically significant when it does occur.

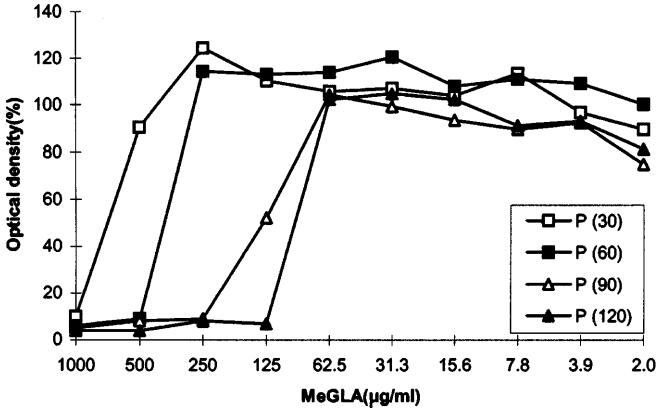

Fig. 1 MGHU-1 optical density (viable cell mass), expressed as a percentage of controls, measured 5 days after gamma-linolenic acid (GLA) exposure lasting 30, 60, 90 or 120 min. There is progressive enhancement of meglumine-GLA (MeGLA) cytotoxicity as the drug exposure time increases

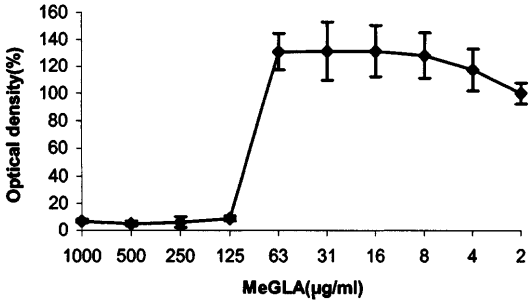

Fig. 2 RT112 optical density (viable cell mass), expressed as a percentage of controls ±1 SD, measured 5 days after GLA exposure lasting 2 h. Values for any concentration ≥125µg/ml (15 wells total) were significantly lower than any other concentration ($P < 0.01$, *t*-test)

Figure 2 represents experiments in which the RT112 cell line was similarly exposed to MeGLA for a period of 2 h. The cytotoxity profile is similar to that of the MGH-U1 cells with an IC > 90 observed at 125 μg/ml.

MTT assay immediately following MeGLA exposure

Figure 3 illustrates experiments in which the cytotoxic effects of MeGLA were assayed immediately after drug exposure to assess the immediate as opposed to a delayed effect. In plates assayed immediately after drug exposure, the IC > 90 was 133 μg/ml. This is similar to the result in experiments in which the plates were allowed to incubate for 5 days prior to assessing cytotoxic effects. The shift to no cytotoxic effects is again a steep slope.

Me-GLA cytotoxicity in resistant tumour cell lines

Figure 4 illustrates experiments in which the doxorubicin-resistant MGH-U1 cells were exposed to Me-GLA for a period of 2 h. The IC > 90 was recorded at 125 μg/ml, which is similar to the finding for the doxorubicin-sensitive parental cell line. Figure 5 demonstrates the cytotoxicity profile of MeGLA on the cisplatin-resistant clone of the RT112 cell line. The cytotoxicity profile is similar to that of the parental line, with an IC > 90 recorded at 125 μg/ml. The previously reported increased sensitivity of resistant lines to GLA is not confirmed.

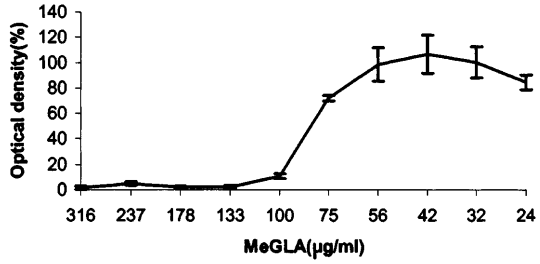

Fig. 3 Immediate cytotoxic effects of MeGLA on MGHU-1 cells. The MTT assay was carried out immediately after drug exposure in three separate plates. The optical density (viable cell mass) is expressed as a percentage of controls ±1 SD. Values for any concentration ≥100 μg/ml (15 wells total) were significantly lower than any other concentration ($P < 0.01$, *t*-test)

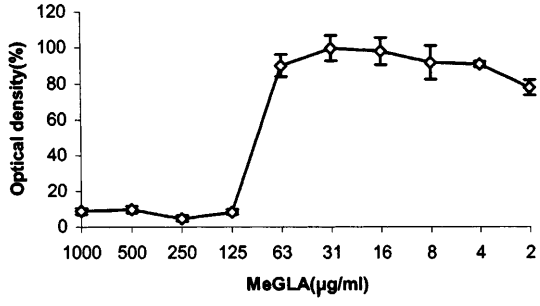

Fig. 4 Cytotoxic effects of Me-GLA on a doxorubicin-resistant strain of MGHU-1 cells. The optical density (viable cell mass) after 5 days of incubation is expressed as a percentage of controls ±1 SD. Values for any concentration ≥125 μg/ml (15 wells total) were significantly lower than any other concentration ($P < 0.01$, *t*-test). The cytotoxicity profile is similar to that of the sensitive strain of MGHU-1

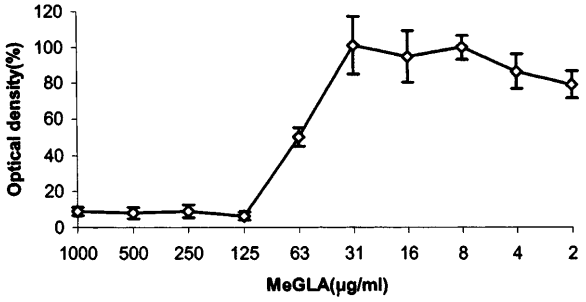

Fig. 5 Cytotoxic effects of Me-GLA on a cisplatin-resistant strain of RT112 cells. The optical density (viable cell mass) after 5 days of incubation is expressed as a percentage of controls ±1 SD. Values for any concentration ≥125μg/ml (15 wells total) were significantly lower than any other concentration ($P < 0.01$, *t*-test). The cytotoxicity profile is similar to that of the sensitive strain of RT112

In vivo tolerance

Animals receiving 0.5 ml of MeGLA at a concentration of 2.5 mg/ml recovered synchronously with saline control rats. The first void urine following the instillation was clear of blood. In subsequent days the MeGLA-treated animals behaved normally and showed no loss of well-being.

Figure 6 is a composite photomicrograph showing control, day 1, day 2 and day 5 bladder histology. Bladders examined at days 1 and 2 following MeGLA instillation at a dose of 2.5 mg/ml revealed mild oedema of the submucosal layers. Histologically, the mucosa remained intact with no evidence of ulceration. At day 5, the submucosal oedema was fully resolved and the day 7 and day 14 animals had normal bladder histology. Urine microscopy revealed no evidence of a urinary tract infection. None of the animals died during the course of the tolerance study.

Discussion

The use of essential fatty acids in the study of malignantly transformed cell lines is well established. Most of these studies have shown these fatty acids to have good cytotoxicity profiles. The sensitivity of bladder cancer cell lines to GLA has only been reported for the unstable and clinically irrelevant methyl ester of the agent [6] and with continuous application over several days.

The intravesical use of GLA in the treatment of superficial bladder cancer is an attractive proposition because the agent appears to have most therapeutic potential when applied in an essentially topical manner

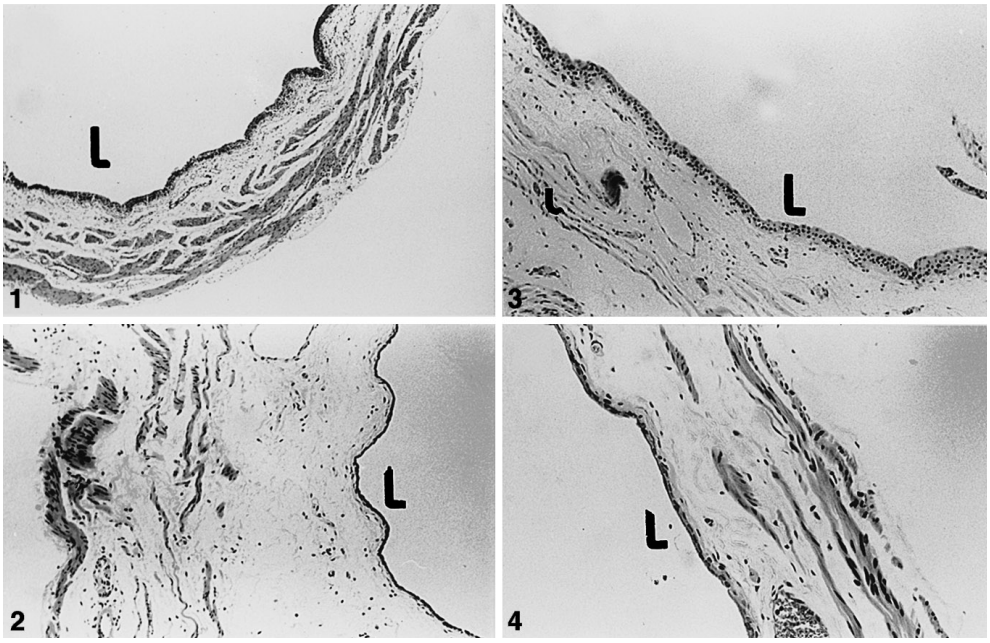

Fig. 6 Composite photomicrographs (× 200) of bladder sections stained with H&E. Specimens were obtained from control (*1*), and rats on day 1 (*2*), day 2 (*3*) and day 5 (*4*) following instillation of 2.5 mg/ml MeGLA. There is submucosal oedema on day 1 that is resolving by day 2 and has fully resolved by day 5

[5]. The application is, however, dependent on the sensitivity of tumour cells at short drug exposure times, as well as the tolerance of the bladder at therapeutic doses. Clinically applicable formulations of GLA include the lithium salt and a methyl glutamine (meglumine) conjugate. The former is being assessed as a parenteral agent, and meglumine-GLA (MeGLA) as a topical preparation, largely because the first use, against glioma, precludes the presence of lithium ions in high concentration. However, MeGLA is supplied at lower concentrations (5 mg/ml) than lithium GLA (27.5 mg/ml).

The in vitro sensitivity of the urothelial tumour cell lines MGH-U1 and RT112 at short drug exposure times has been established by this study using an MTT assay. Viability testing is a vexed topic and each type of test measures a different aspect of survival. Although MTT reduction is not a direct cell counting method it "is now widely chosen as the optimal endpoint" [8]; the factors affecting the assay are discussed by this author. A recent comparative study by Basha et al. [1] supports these conclusions. As we employ no late washing step, no loss of formazan occurs at this stage. Cytotoxicity curves show steep transitions between minimal and maximal cell killing and little effect on results according to the length of incubation between drug exposure and MTT assay. This is suggestive of catastrophic physical membrane disruption due to fluidity changes or surfactant effects rather than metabolic cytotoxicity. MeGLA was cytotoxic to a monolayer of tumour cells at concentrations equal to or greater than 125 μg/l.

The tolerance of rat bladder to intravesical GLA at a dose of 2.5 mg/ml is excellent, as shown in this study. Histological changes are minimal and resolve quickly. There is no alteration whatever in the behaviour or well-being of the animals after treatment over and above those associated with a light general anaesthetic. This dose is an order of magnitude higher than that necessary to exert maximum cytotoxicity in vitro.

MeGLA shows a near-identical cytotoxicity profile between parental, epirubicin-sensitive MGH-U1 cells and the MDR variants induced by doxorubicin and mitomycin. This is of particular interest as multidrug resistance is considered to be a major clinical problem with these intravesical chemotherapeutic agents [13]. Non-MDR, cisplatin-resistant RT112 cells yield near-identical results.

Overall, the results confirm that GLA is a feasible intravesical agent for superficial bladder cancer although the duration of drug exposure should be at least 1 h for optimal effect. The adherent monolayer model used in this study cannot evaluate how well MeGLA penetrates a multilayered arrangement of tumour cells, neither does it address the interesting question of the effect of substrate on the production of protective uroplakins [15]. Our intention, therefore, is to pursue the matter further using organ culture and xenografting techniques. The evidence in the literature that GLA-treated cells can show enhanced uptake of conventional agents [6] points towards assessment of the product for combination as well as sole use.

Acknowledgements The authors gratefully acknowledge support, both financial and material, from the Research and Development Directorate of the South & West Regional Health Authority and Scotia Pharmaceuticals.

References

1. Basha G, Yap P, Penninckx F (1996) Comparative study of classical, colorimetric and immunological staining methods for the assessment of tumor-cell viability. Tumour Biol 17:354–361

2. Begin ME, Das UN, Ells G, Horrobin DF (1985) Selective killing of human cancer cells by polyunsaturated fatty acids. Prostaglandins Leukot Essent Fatty Acids 19:177–86
3. Booyens J, Engelbrecht P, Le Roux S, Louwrens CC, Van der Merwe CF, Katzeff IE (1984) Some effects of the essential fatty acids linoleic acid and alpha-linolenic acid and of their metabolites gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and of prostaglandins A_1 and E_1 on the proliferation of human osteogenic sarcoma cells in culture. Prostaglandins Leukot Med 15:15–33
4. Botha JH, Robinson KM, Leary WP (1983) Parenteral gamma-linolenic acid administration in nude mice bearing a range of human tumour xenografts. S Afr Med J 64:11–12
5. Das UN, Prasad VVSK, Raia Reddy D (1995) Local application of gamma-linolenic acid in the treatment of human gliomas. Cancer Lett 94:147–55
6. Davies CL, Duffy PM, MacRobert AJ, Loizidou MC, Cooper AJ, Taylor I (1996) Localization of anthracycline accumulation in sensitive and resistant urothelial tumor cell lines. Cancer Detect Prevent 20:625–633
7. Dippenaar N, Booyens J, Fabbri D, Katzeff IE (1982) The reversibility of cancer: evidence that malignancy in melanoma cells is gamma-linolenic acid deficiency-dependent. South Afr Med J 62:505–509
8. Freshney RI (1994) Measurement of viability and cytotoxicity. In: Culture of animal cells, 3rd edn. Wiley, New York, pp 296–298
9. Horrobin DF (1980) The relevance of cyclic AMP, calcium, essential fatty acids and prostaglandin E_1. Med Hypoth 6:469–486
10. Leary WP, Robinson KM, Booyens J, Dippenaar N (1982) Some effects of gamma-linolenic acid on cultured human esophageal carcinoma cells. S Afr Med J 62:681
11. Masters JR, Hepburn PJ, Walker L, Highman WJ, Trejdosiewicz LK, Povey S, Parkar M, Hill BT, Riddle PR, Franks LM (1986) Tissue culture model of transitional cell carcinoma: characterization of twenty-two human urothelial cell lines. Cancer Res 46:3630–3636
12. Morisaki N, Sprecher H, Milo GE, Cornwell DG (1982) Fatty acid specificity in the inhibition of cell proliferation and its relationship to lipid peroxidation and prostaglandin biosynthesis. Lipids 17:893–899
13. Nakagawa M, Emoto A, Nasu N, Hanada T, Kuwano M, Cole SPC, Nomura Y (1997) Clinical significance of multi-drug resistance associated protein and P-glycoprotein in patients with bladder cancer. J Urol 157:1260–1265
14. Sircar S, Cai F, Begin ME, Weber JM (1990) Transformation renders mdr cells more sensitive to polyunsaturated fatty acids. Anticancer Res 10:1783–1786
15. Smith B, Kennedy W, Trejdosiewicz LK, Selby PJ, Southgate J (1997) Induction of uroplakin 2 gene expression by differentiating normal human urothelial cell cultures. Br J Urol (in press)
16. Southgate J, Pitt E, Trejdosiewicz LK (1996) The effects of dietary fatty acids on the proliferation of normal human urothelial cells in vitro. Br J Cancer 74:728–734
17. Weber JM, Sircar S, Begin ME (1994) Greater sensitivity of human multidrug resistant (mdr) cancer cells to polyunsaturated fatty acids than their non-mdr counterparts. J Natl Cancer Inst 86:638–639